Activation of a Ste20/PakA protein kinase occurs in the dermatophyte *Trichophyton rubrum* through alternative splicing

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This present work aims to describe a probable mechanism for activation of a Ste20/PakA protein kinase through intron retention process, modulated by the antifungal agent undecanoic acid (UDA). Ste20/PakA belongs to the *Trichophyton rubrum* mitogen-activated protein kinase (MAPK) pathway. For this, approximately 1×10^6 conidia/mL from *T. rubrum* strain CBS 118892 was inoculated in Sabouraud medium and pre-cultured at 28°C for 96 h under constant agitation. For the high-throughput sequencing analysis, the mycelia were aseptically transferred to RPMI 1640 media (Gibco, USA) in the absence or presence of 17.5 µg/mL UDA (Sigma, USA), which corresponds to 70% of its minimum inhibitory concentration (MIC). These cultures were incubated for 3 or 12 h at 28°C under constant agitation and the mycelia used for RNA extraction. For the RT – qPCR analyses, after the pre-culture step, the mycelia were aseptically transferred to a fresh Sabouraud medium in the absence or presence of 70% and 100% of its MIC, and then incubated for 12 h at 28°C under constant agitation. The total RNA from all samples was isolated through the TRIzol RNA kit (Invitrogen Life Technologies), and used for the synthesis of cDNA with the TruSeq RNA library Kit (Illumina, USA). The libraries were sequenced on Illumina HiSeq 2000 system (Illumina, USA). Intron retention analysis was performed through *ad hoc* Perl scripts, being identified in the reference genome, aligned against the sequenced libraries, and analysed for each retained intron. RT-PCR was used to confirm the properties of genes of interest, using primers flanking the region of intron 1 (Fig. 1A), generating fragments of 336 bp and 198 bp, in amplifications with and without intron retention, respectively. (Fig. 1B).

Fig. 1: Intron retention analysis of the *T. rubrum* *ste20* kinase gene. **A** - Graphic representation of the *ste20* gene indicating the annealing site (blue and orange arrows) of the primer set used for amplification of the intron-1 region. **B** - Agarose gel electrophoresis the *ste20* gene amplification product. **C** - RT – qPCR analysis of the relative intron-1 expression in 3 h, 12 h, and 24 h after UDA incubation. **Legends:** Cont.: Control condition; Gen.: Genomic DNA; MM: Molecular Marker.

Gene expression was quantified by qPCR with the StepOnePlus Real-Time PCR system (Applied Biosystems, US). *In silico* analysis indicated that the *ste20/pakA* mRNA in *T. rubrum* encodes for a protein kinase with 970 amino acids, presenting the catalytic domain in the C-terminal region, and an auto-inhibitory domain (Cdc42/Rac interactive binding - CRIB) in the N-terminal (Fig. 2A). In *Saccharomyces cerevisiae*, Ste20p is transiently activated by the Rho GTPase Cdc42p, binding to the CRIB domain in response to moderate stress, and also activated by caspase cleavage during apoptotic stress in mammals. Our data suggest that retention of the intron-1 generates a premature stop codon in its mRNA, followed by a new start codon and a subsequent sequence encoding for a protein that exhibits the catalytic portion with a high degree of homology with the native one, but free from the CRIB domain (Fig. 2B). The data also indicated that the retention level was modulated by the presence of UDA (Fig. 1C). Thus, we hypothesize an alternative mechanism, analogous to the caspase 3 cleavage, for the activation of PakA by intron retention in *T. rubrum*, which can be modulated by UDA, probably for a faster transcriptional response through the MAPK pathway.

Fig. 2: Graphic representation of the *ste20* gene, gene transcription, and gene translation products. **A** - Graphic representation of the *ste20* gene indicating the gene structures with five exons and four introns; graphic representation of the *ste20* mRNA product normally processed; and graphic representation of the Ste20 protein with its main domains, respectively. **B** - Graphic representation of the *ste20* gene indicating the gene structures with five exons and four introns; graphic representation of the *ste20* mRNA product with intron 1 retention, and graphic representation of the two polypeptides products formed from the *ste20* translation, with its respectively protein domains.